Reference: *Biol. Bull.*, **138**: 334–343. (June, 1970).

ISOLATION, LOCALIZATION AND BIOSYNTHESIS OF CRASSIN ACETATE IN *PSEUDOPLEXAURA POROSA* (HOUTTUYN)[1]

JOHN R. RICE, CONSTANTIN PAPASTEPHANOU AND DAVID G. ANDERSON

Department of Biochemistry, University of Miami, School of Medicine, Miami, Florida 33152

Crassin acetate was first isolated by Ciereszko, Sifford and Weinheimer (1960), from the whole dried gorgonian *Pseudoplexaura porosa* (Houttuyn). Ciereszko (1962), later reported that this diterpene lactone was present in very high concentration in the symbiotic zooxanthellae isolated from this gorgonian. We have been studying sesquiterpene hydrocarbon biosynthesis in zooxanthellae isolated from *P. porosa*. A major difficulty in isolating pure zooxanthellae is in separating them from a large volume of colorless micro-crystals. The objectives of the present study were to check the identity of the crystals with crassin acetate, to eliminate the possibility that their extracellular occurrence is an artifact of the isolation procedure, to determine the histological relationship of the crystals, the zooxanthellae, and the host tissue, and to study the metabolism of crystalline crassin acetate.

Materials and Methods

Isolation of crassin acetate crystals from Pseudoplexaura porosa

P. porosa was collected from the coral patch reefs known as Bache Shoal just off Elliott Key, Florida, transported in sea water, and processed immediately on return to land.

Segments of gorgonian 3 to 4 cm long were homogenized in a Waring Blendor at full speed for 10 seconds with approximately twice their volume of sterile 50 per cent sea water, a medium which was used throughout this preparation. The medium was sterilized by passage through a 0.45 μ pore size Millipore filter. Large fragments and gorgonian stipes were removed by a single passage through cheese cloth. After the strained homogenate was centrifuged at 3000 g in 250 ml centrifuge bottles for 15 minutes, the supernatant was discarded and the buff-colored surface layer was removed by gentle swirling with medium. The remainder of the pellet was resuspended in a 20-fold volume of medium and centrifugation and washing was repeated two more times.

The final pellet was resuspended in six times its volume of medium, immediately transferred to 12 ml centrifuge tubes and centrifuged at 2000 g in a swing head for ten minutes. The four layered pellet which formed was separated into fractions by differential resuspension using a syringe-controlled pipette. In general, it was technically simple to resuspend the bulk of a layer without introducing substantial contamination from the layer immediately below. The shallow

[1] Work supported by research grant GM-12229 from the National Institute of General Medical Science, Public Health Service.

top layer (debris) was resuspended by a gentle stream and discarded. Progressively stronger streams from the pipette served to resuspend the second (zooxanthellae) layer and the third (crystalline) layer. Fractions from these two layers were reserved as a predominantly zooxanthellar fraction, a mixed fraction, and a crystalline fraction. The fourth (spicule) layer was discarded. The reserved fractions were repeatedly processed via centrifugation and differential resuspension until the zooxanthellae and crystals were minimally contaminated with each other.

Small scale extraction of crassin acetate (radiochemical experiments)

Fresh *P. porosa,* about 25 grams, was cut into 3 cm lengths and extracted three times in a Waring Blendor with a total of 250 ml of 50% petroleum ether in methanol. Two grams of Hyflo-SuperCel were added as a filter aid for the first extraction. The mixture was filtered under vacuum through Whatman No. 1, and the filter cake was re-extracted in the Blendor. The fourth and final extraction employed 50 ml of methanol. The combined extracts formed two layers, the epiphase petroleum ether and the hypophase approximately 80% methanol in H_2O. After phase separation the petroleum ether phase was washed twice with 80% methanol in H_2O, and the methanol phase with petroleum ether. Each wash was added to its corresponding fraction. Concentration of the methanol rich fraction gave an oily residue. Methanol washes of the residue were combined with the soluble methanol fraction and decolorization achieved by boiling with acid-washed charcoal and filtering. Addition of an equal volume of warm water followed by cooling crystallizes crassin acetate. Recrystallizations and further decolorizations, if required, proceeded via the above procedure using a minimal amount of methanol as solvent.

Large scale extraction of crassin acetate

Colonies of *P. Porosa* were dried in the open air. When constant weight was achieved, the cortex was stripped from the stipe and reduced to a coarse powder in a Waring Blendor. Five pounds of the dried powder were placed in the continuous extractor described by Ciereszko (1966) and subjected to continuous extraction with petroleum ether so as to remove the bulk of pigments and some lipids that complicate the crystallization of crassin acetate. When the extractant overflow was barely tinted, extraction was interrupted, the extracted cortex was dried at room temperature and then subjected to a single methanol extraction by a batch process, 500 ml per 100 grams of dried cortex.

While by no means colorless, this extract produced purified crassin acetate crystals when the volume was reduced and water added to incipient crystallization, followed by cooling. Decolorization with charcoal and recrystallization from methanol-water resulted in pure crassin acetate, melting point 141° C.

Infrared spectroscopy

For infrared spectroscopy a portion of the crystals isolated in unmodified form by homogenization in 50 per cent sea water and centrifugation were washed essen-

tially free of the few remaining zooxanthellae on a coarse sintered-glass filter and then compressed in a KBr pellet. The spectrum was taken on a Beckman IR-8 infrared spectrophotometer.

Histological preparation

Fresh segments of gorgonian were relaxed for 2 to 5 minutes in 1.2% $MgCl_2$, fixed for 12 hours in 10% acetate-buffered formalin, pH 6.5–8.0, and decalcified for 12 hours in 0.5 *M* sodium ethylenediaminetetraacetate (EDTA), pH 8.3. Specimens were embedded in 6% gelatin and frozen sections cut at 10 μ on a CO_2 freezing microtome. Some sections were stained by flotation in 0.1% toluidine blue for 15 seconds and briefly washed in water. Other sections were not stained. All sections were permanently mounted using Zwemer's glycrogel mounting medium.

Acid treatment of the cortex

Four-inch segments of gorgonian tip were dried at room conditions to constant weight. Each segment was exposed to 10 ml of 2 *N* HCl for three days, after which the remaining solid material was washed with distilled water on weighed filter paper. The filter paper and gorgonian remains were dried for three days under room conditions and weighed.

Chromatography of crassin acetate

Thin layer chromatography employed aluminum oxide and silica gel. Precoated plates obtained from Brinkmann Instruments, Westbury, New York, consisted of aluminum oxide (Type E) with fluorescent indicator F_{254}, Merck AG 5751/0025, and silica gel with F_{254}, Merck AG 5714. A detection spray (Attaway, Wolford and Edwards, 1965) of 5% vanillin in concentrated H_2SO_4 gives a gray-violet spot with crassin acetate on heating at 110° C for 10 minutes. With this detection technique, thin layer plates with F_{254} provide a whiter and more uniform background than plates lacking the indicator. Silica gel plates were run with 20% acetone in benzene. The R_f of crassin acetate in this system is 0.49. Twenty per cent dioxane in benzene on aluminum oxide gave a crassin acetate R_f of 0.59. Aluminum oxide plates with acetone:benzene (1:4) gave an R_f of 0.75.

In column chromatography of crassin acetate, an aluminum oxide (Merck for chromatography) column 1.4 × 11 cm was developed with stepwise increases of ethyl acetate in petroleum ether. Crassin acetate is eluted by 70% ethyl acetate.

Colorimetric determination of crassin acetate

In a direct method, crassin acetate in methanol was allowed to stand at room temperature with 3 ml of 5% freshly prepared vanillin in conc. H_2SO_4. The product has an absorption maximum at 540 mμ and was read at this wave length against a reagent blank containing the standard volume of methanol. Absorbance is linear within the range of 10 to 50 μgrams of crassin acetate. A disadvantage of this method arises from the high blank caused by methanol. A second colorimetric procedure below avoids this difficulty.

Colored spots on the developed and sprayed thin layer chromatogram were scraped off and eluted with small multiple volumes of acetone, adjusted to 1.0 ml and read at 540 mμ. Standardization was accomplished by the same technique. While results are somewhat erratic and routine standardization is required, this technique is useful in the range of 20 to 200 μgrams of crassin acetate.

Incubation with radioactive substrates

Radioactive carbonate in the form of $Na_2{}^{14}CO_3$, 6.4 μc/μmole was purchased from New England Nuclear, and $^{14}CH_3COOH$, 51 μc/μmole as the sodium salt was purchased from Amersham-Searle. Incubations were performed in stoppered Pyrex 500 ml reagent bottles. The radioisotope in 0.2 ml of 0.05 *M* KOH was placed in the bottles on land and taken to the reef area. To each bottle 400 ml of sea water from the collection site was added. A single colony of *P. porosa* was collected and a 10 cm segment cut from each tip. Seven such segments were placed in each sea water-isotope solution, sealed, and exposed to direct sunlight for a period of two hours. The gorgonian segments were then processed for isolation of crassin acetate. These seven gorgonian segments represent about 25 grams wet weight and seven grams dry weight. Two experiments were carried out using two different colonies. In experiment I the specific radioactivity of the carbonate ^{14}C was 0.11 μc/μmole and that of acetate 2-^{14}C was 0.20 μc/μmole; the total radioactivity was 125 μc/liter and 10 μc/liter, respectively. In experiment II the specific radioactivity of the carbonate ^{14}C was 0.165 μc/μmole and that of acetate 2-^{14}C was 50 μc/μmole; the total radioactivity was 182 μc/liter and 625 μc/liter, respectively. The value for CO_3^{2-} and HCO_3^- used in calculating specific activity was 2.31 milliequivalent/liter of sea water.

Radioactivity measurements

Scintillation methods were employed for radioactivity measurements. Two instruments, a Packard Tricarb Liquid Scintillation Spectrometer Model 4312 and a Beckman Liquid Scintillation System Model LS were employed. In both instruments, windows which counted ^{14}C with about 75% efficiency were used. Efficiency was routinely checked with internal standards dependent on the scintillation medium. Toluene with PPO and POPOP served in counting toluene-miscible samples, while naphthalene-dioxane, PPO and POPOP was used for aqueous samples. Suspected quench was detected and corrected for by use of internal standards.

Determination of labeling pattern, acetate vs. *crassin*

Distribution of radioactivity between the crassin ring and the acetate moiety of crassin acetate is determined by acid hydrolysis of the ester, separation of acetic acid and crassin by lyophilization in a closed system, and finally, counting the residual crassin and products derived therefrom and the distillate, acetic acid. Hydrolysis of a weighed sample of crassin acetate of constant specific radioactivity is carried out with 2 *N* HCl in a sealed ampoule at 110° C for 17 hours. The ampoule is transferred to one arm of a U-tube and broken. This arm is then

chilled by immersing in solid CO_2-acetone. The system is evacuated to a pressure of 0.1 mm Hg and then sealed. The second arm of the U-tube is placed in the cold bath and the contents of the other arm allowed to warm to room temperature. All volatile components of the hydrolyzate will thus be transferred to the second arm while the non-volatile components remain behind.

Results and Discussion

Infrared absorption studies

Comparison of the infrared absorption spectrum of the unmodified crystals isolated from *P. porosa* with the spectrum of authentic crassin acetate (see Fig. 1) reveals that the spectra are essentially identical. Furthermore, it is evident that the unmodified crystals isolated directly from the homogenate of *P. porosa* in sea water are crassin acetate of a high degree of purity. Since the crystal fraction contains some visible contamination, recrystallization is necessary to obtain an adequate melting point. One recrystallization is usually sufficient to give material with a constant melting point of 140–141° C. This compares with the melting point of 138–140° C reported by Weinheimer, Schmitz and Ciereszko (1968), who also showed that crassin acetate is based on the 14 carbon cembrane ring (Fig. 2).

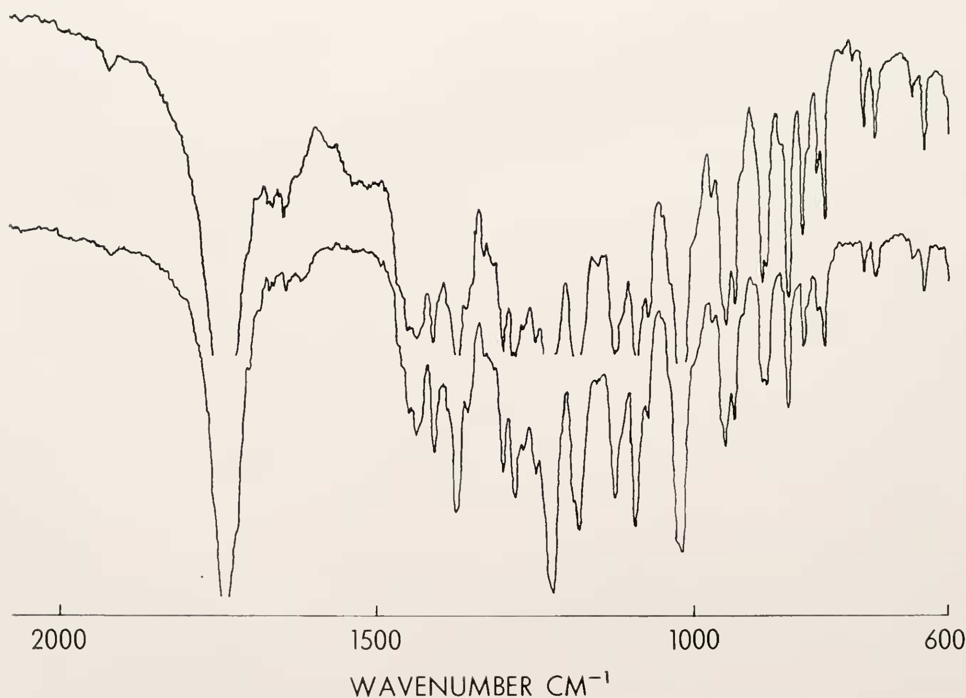

FIGURE 1. Infra red spectra; isolated unmodified crystals (upper curve) compared with authentic crassin acetate (lower curve).

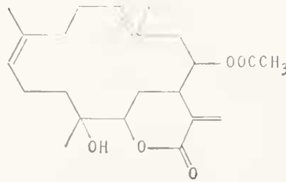

FIGURE 2. Structure of crassin acetate, Weinheimer *et al.* (1968).

Histological studies

Ciereszko (1962), suggested that crassin acetate is produced by and contained within the symbiotic zooxanthellae of the gorgonian. The occurrence of free crystals of crassin acetate might, therefore, be an artifact of the isolation process. Cover slip crush preparations of individual polyps of *P. porosa* readily demonstrated that the crystals identified as crassin acetate are indeed extracellular to the zooxanthellae. However, sectioning of fixed material was necessary to establish precise anatomical relationships. Most standard fixation and embedding techniques dissolve crassin acetate, hence the procedure detailed in the materials and methods section was adopted. Even then, an absolute minimum of fixation and decalcification time was necessary. Crassin acetate is slowly soluble in buffered formalin and EDTA probably by conversion of the lactone to the water soluble carboxylate.

Toluidine blue stains zooxanthellae and the stipe of the gorgonian a deep green. The remainder of the gorgonian tissues stain varying intensities of blue with the exception of certain cell types, particularly those lining the stomodeum of the polyps, which stain magenta. Crystals of crassin acetate and the organic matrix of the calcium carbonate spicules of the gorgonian do not stain. A typical photomicrograph of an unstained section (see Fig. 3), shows the relationship of host tissue, zooxanthellae and crystals of crassin acetate. Examination of the photomicrograph indicates a close association of crassin acetate crystals with the symbiotic zooxanthellae found in the host endoderm. Although the crystals may be found free in the cavities and longitudinal canals of the polyps, they are found in greatest profusion embedded in host tissues which contain zooxanthellae. Furthermore, crystals are absent from zooxanthellae-free tissue. This leads us to conclude that crassin acetate is most likely produced by zooxanthellae, or by the interaction of the host endoderm-zooxanthellae complex.

Intracolonial distribution of crassin acetate

Studies of the distribution of crassin acetate along the length of the colony from base to tip employing the semiquantitative thin-layer technique and the direct colorimetric measure reveal no striking variations in the concentration of crassin acetate in the gorgonian cortex. This suggests a rapid synthesis followed by quiescence rather than continued slow synthesis. An alternative explanation would be turnover of the crassin acetate, a possibility which we are further exploring. However, the cortex represents larger increments of the total colony with progress along the stipe from base to tip. Thus considering the whole

colony including the stipe, the concentration of crassin acetate increases from base to tip.

Concentration of crassin acetate

The concentration of crassin acetate in the *P. porosa* cortex was estimated by two methods: (1) the semi-quantitative thin-layer technique and (2) weighing the crassin acetate purified and isolated from a known weight of dried cortex. Both methods gave similar results. Averaging six determinations on three samples shows 4.4% of the dry cortex and 1% of the whole dried colony as the minimum concentration of crassin acetate. No attempt was made to estimate losses which were probably large.

A substantial portion of the cortex is made up of calcium carbonate spicules. Therefore we used dilute HCl treatment to dissolve the spicules and found that 39 to 41% of the dried tip cortex (with or without the central stipe) was not soluble in acid. Neglecting the possibility that acid treatment solubilized substantial amounts of material in addition to calcium carbonate, we can conclude that crassin acetate represents at least 10% of the organic substance (acid insoluble) of the dried gorgonian cortex.

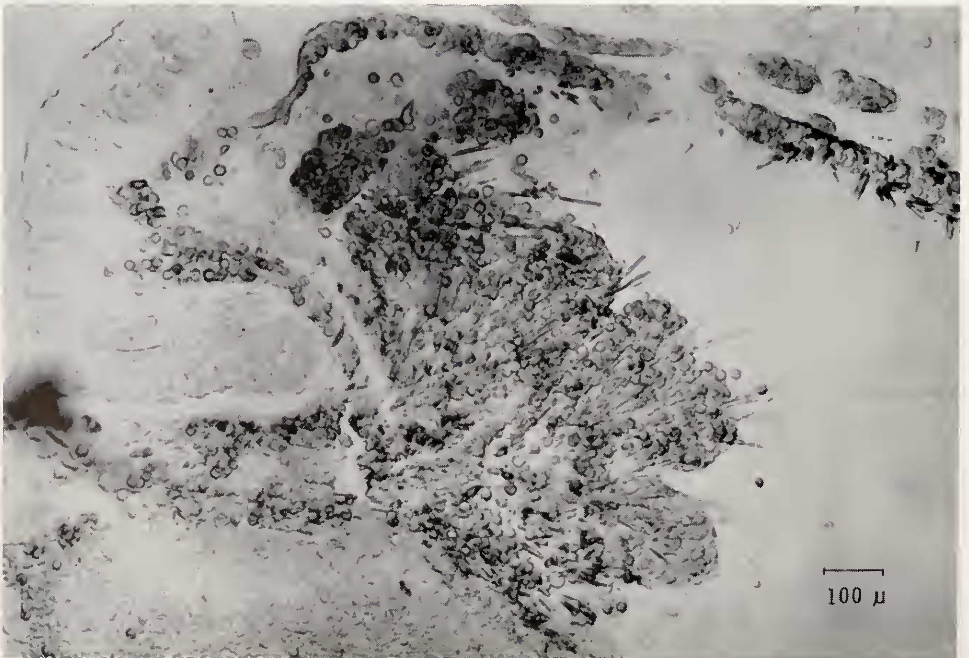

FIGURE 3. Unstained decalcified frozen section of *P. porosa* polyp. Elongate forms are crassin acetate crystals, many project into the digestive cavity. Darker circular bodies are zooxanthellae located primarily in host gorgonian endoderm. Paler tissues which are zooxanthellae free are largely host exoderm.

Metalobism of crassin acetate

When intact tips of *P. porosa* colonies are incubated in sea water with ^{14}C carbonate or acetate, a substantial portion of the label is incorporated into the organic material of the gorgonian-zooxanthellae complex. However, net incorporation into crassin acetate is small. From Table I it is apparent that crystallization of the isolated crassin acetate quickly achieves constant specific radioactivity in spite of the wide range of radioactive products which must have formed. It is also apparent that acetate is superior to carbonate as a crassin acetate precursor by approximately ten-fold.

TABLE I

*Labeling of crassin acetate in cpm/µmole**

Crystallization Number	Carbonate ^{14}C		Acetate 2-^{14}C	
	Experiment I	Experiment II	Experiment I	Experiment II
1	4.5	—	—	—
2	4.1	—	43.2	—
3	3.2	1.1	47.3	925
4	3.7	1.2	46.2	1010
9	3.3	—	—	—

* In experiment I, tips from a single gorgonian colony were used. In experiment II, a different single colony served as a source of tips. See "Materials and Methods" for details.

In some labeling experiments high specific activity precursors were employed. In addition carbonate and acetate enter into a host of pathways. Because specific coprecipitation might account for the swift attainment of constant specific radioactivity a further test system employed column chromatography. Fractions were collected over the course of elution, evaporated, weighed and the radioactivity of the total sample determined. Results are presented in Table II.

Except for the first fraction, satisfactory specific radioactivity is maintained across the peak. We conclude that crassin acetate has been labeled in these experiments.

Incorporation of acetate radioactivity into crassin acetate might merely reflect the exchangeability of the acetate ester moiety. Release by hydrolysis and trapping

TABLE II

*Distribution of radioactivity in crassin acetate fraction**

Fraction	Wt. of crassin acetate	cpm	Specific radioactivity cpm/mg
26	2.7	175	64
27	11.5	1276	110
28	19.0	2374	124
29	7.3	846	122
30	2.1	214	102

* See "Materials and Methods" for details. Crassin acetate applied to the column had an activity of 122 cpm/mg.

of the ester acetate revealed that a substantial portion, but less than half, of the label in crassin acetate was present in the acetate ester moiety. Two experiments gave essentially identical results; 960 cpm in released acetic acid from 2480 cpm in the original crassin acetate, and 872 cpm in acetic acid from 2010 cpm in the original crassin acetate, 38.6 and 43.4% of the label in the acetate moiety, respectively. We can conclude that a substantial portion of the label in crassin acetate arises by ester exchange, or esterification of newly formed crassin. The crassin ring is also labeled, however, since a large portion of the label is found in the residue remaining after hydrolysis. Further experiments to define the labeling pattern in crassin acetate, specifically, the crassin ring, are in progress.

These experiments have shown that the gorgonian-zooxanthellar complex has the capability of synthesizing crassin acetate *de novo*. The possibility existed that crassin acetate was accumulated or modified, and segregated by feeding and digestion processes. This is obviously not the case. Our various attempts to demonstrate biosynthesis of crassin acetate by isolated *P. porosa* zooxanthellae were unsuccessful. Thus, in conjunction with the present results, our hypothesis that crassin acetate is formed as a joint venture by gorgonian and zooxanthellae is still tenable.

The extremely high level of crassin acetate found in *P. porosa* accentuates the problem of function of this diterpene. Ciereszko *et al.* (1960), have found that crassin acetate is highly toxic to a number of marine vertebrates, as well as being antiprotozoal and antibacterial; they, therefore, conclude that crassin acetate may have a protective function. Most personnel in our laboratory who have worked with *P. porosa* homogenates have experienced a toxic reaction about the eyelids resembling a pruritic contact eczematous dermatitis. The untested assumption is that the reaction is caused by microcrystalline crassin acetate.

Aside from toxicity no function of crassin acetate has been demonstrated. It is tempting to speculate that it or other terpenes of zooxanthellae may serve as metabolic energy stores for the zooxanthellae or the gorgonian host.

We wish to thank Drs. A. J. Weinheimer, Leon S. Ciereszko and Tom K. B. Karns, Department of Chemistry, The University of Oklahoma, for providing the infrared absorption spectra as well as results of structural studies prior to publication; Dr. Frederick M. Bayer of the Rosenstiel School of Atmospheric and Marine Science, University of Miami, for aid in gorgonian identification; and Dr. Irwin Berman and Mrs. Dorothy Stokes of the Department of Anatomy for aid and advice in the histological studies. Requests for reprints should be addressed to Dr. David G. Anderson.

Summary

1. The particulate portion of a homogenate of the gorgonian *Pseudoplexaura porosa* (Houttuyn) can be separated into fractions by repeated centrifugation and differential resuspension.

2. One of these fractions is crystalline and has been identified as crassin acetate, a diterpene lactone, by the infrared absorption of the original crystals and by melting point determination after recrystallization.

3. Histological sections demonstrate that crassin acetate crystals are extracellular, in part, to the gorgonian tissues, and *in toto*, to the symbiotic zooxanthellae of this organism. However, the crystals occur in association with gorgonian endoderm containing zooxanthellae, thus suggesting that crassin acetate is a product of host-zooxanthellae interaction.

4. Quantitative determinations of crassin acetate show a minimum concentration of 10% of the organic dry weight of the cortex. It was also found that cortex from both youngest and oldest parts of the gorgonian colony contain essentially equal concentrations of crassin acetate.

5. Both CO_2 and acetate are precursors of the diterpene, acetate being about ten times more efficient.

LITERATURE CITED

ATTAWAY, J. A., R. W. WOLFORD AND G. I. EDWARDS, 1965. Determination of esters by thin layer chromatography. *Anal. Chem.*, **37**: 74–76.

CIERESZKO, L. S., D. H. SIFFORD AND A. J. WEINHEIMER, 1960. Chemistry of Coelenterates, I. Occurrence of terpenoid compounds in Gorgonians. *Ann. New York Acad. Sci.*, **90**: 917–919.

CIERESZKO, L. S., 1962. Chemistry of Coelenterates. III. Occurrence of antimicrobial terpenoid compounds in the zooxanthellae of alcyonarians. *Trans. New York Acad Sci.*, **24**: 502–503.

CIERESZKO, L. S., 1966. A versatile continuous percolator-extractor. *J. Chem. Education*, **43**: 252.

WEINHEIMER, A. J., F. J. SCHMITZ AND L. S. CIERESZKO, 1968. Chemistry of coelenterates—VII, pp. 135–140. *In:* H. D. Freudenthal, Ed., *Drugs from the Sea.* Marine Technology Society, Washington, D. C.